AD_____

Award Number:
W81XWH-12-1-0496

TITLE:
Uterine-specific knockout of Tsc-2: a mouse model for lymphangioleiomyomatosis

PRINCIPAL INVESTIGATOR:
Stephen R Hammes, M.D., Ph.D.

CONTRACTING ORGANIZATION:
University of Rochester
Rochester, NY

REPORT DATE:
October 2013

TYPE OF REPORT:
Annual Report

PREPARED FOR:
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2013	Annual	30 September 2012–29 September 2013

4. TITLE AND SUBTITLE

Uterine-specific knockout of Tsc-2: a mouse model for lymphangioleiomyomatosis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0496

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Stephen R Hammes
Betty Diamond

E-Mail: Stephen_hammes@urmc.rochester.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Rochester
910 Genesee St STE200
Rochester, NY 14611-3847

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our goal was to develop and characterize a mouse model for the disease lymphangioleiomyomatosis (LAM). This disease is found almost exclusively in women and is due to a mutation in one of the two TSC suppressor genes. Women with LAM develop smooth muscle-like tumors in the lungs that grow progressively, often leading to lung failure. Evidence suggested that these tumors do not originate in the lungs but instead are metastatic from another location in the body. Our hypothesis was that lung LAM tumors were actually metastatic from smooth muscle myometrial cells in the uterus, thus explaining both the appearance of the LAM cells as well as the overwhelming female prevalence. Thus, we created a uterine-specific TSC-2 knockout mouse. These mice developed uterine tumors that were completely dependent on estrogen for growth. Importantly, as mice aged above 30 weeks, 75% of them developed myometrial lung tumors that shared many features consistent with LAM. These data were just published in and featured on the cover of the September, 2013 issue of "Molecular Endocrinology." This is an exciting new model, and we hope to use it to both better understand LAM and to help test novel treatment options for LAM.

15. SUBJECT TERMS
lymphangioleiomyomatosis, uterus, leiomyoma, metastatic lung tumors, estrogen-dependent

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	17	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Uterine-specific knockout of Tsc-2: a mouse model for lymphangioleiomyomatosis

Stephen R Hammes, MD, PhD, University of Rochester
Rochester, NY

Introduction:

Lymphangioleiomyomatosis (LAM) is a rare disease that consists of smooth muscle cell tumors within the lungs that progressively grow, often leading to pulmonary failure and sometimes even lung transplantation. Interestingly, even after transplantation, LAM tumors often return to the lungs, suggesting that LAM cells do not originate in the lung. Two other unusual features of LAM are: 1) LAM is found almost exclusively in women; and 2) LAM cells contain mutations in one of the two TSC genes. To explain all of these observations, we proposed that LAM cells might metastasize from smooth muscle myometrial cells in the uterus that have sustained mutations in one of the TSC genes. To test this theory, we proposed to make a uterine-specific TSC-2 knockout mouse by mating a mouse expressing Cre driven by the progesterone receptor promoter with a mouse containing a floxed TSC-2 gene. Our goal was to: 1) test our hypothesis; 2) create a viable mouse model for LAM that can be used to study LAM physiology as well as to test LAM treatment options; and 3) create a TSC-2 null myometrial cell line that can be used to study molecular mechanisms of LAM proliferation.

Body:

This project has been remarkably successful in the first year of funding from the DOD. Our first task was to mate TSC-2 floxed and PR-Cre mice to create a uterine-specific TSC-2 null mouse. Subtasks included examination of estrus cycling, measurement of hormone levels, examination of the uterine phenotype, examination of the ovarian, pituitary, and hypothalamic regions, examination of the lungs, and a look at the dependence of mTOR signaling for tumor growth. In fact, as seen in the accompanying manuscript, we have completed at all of these tasks. We have used quantitative PCR and Western blot to confirm that we have a uterine-specific TSC-2 null mouse. These mice are chronically in estrus and have normal estradiol levels. Their uteri are markedly enlarged, and older mice develop myometrial tumors (leiomyomas). These tumors are mTORC1-dependent, as treatment with the mTORC1 inhibitor rapamycin abrogate myometrial proliferation. The ovaries, hypothalamus, and pituitary of these mice are normal. Finally, 75% of older uterine-specific TSC-2 null mice develop metastatic myometrial lung tumors, confirming that LAM might indeed originate from the uterus. All of these accomplishment are described in detail in the attached publication (see appendix).

Task 2 was to remove the ovaries from uterine-specific TSC2 null mice and then add back estrogen versus progesterone to see which steroid, if any, promotes myometrial overgrowth. In fact, removal of the ovaries completely abrogated leiomyoma formation, and addition of estradiol, but not progesterone, pellets completely restored tumor formation. Thus, like LAM, myometrial tumor formation in the TSC-2 null uterus requires estradiol signaling. Again, these accomplishments are described in detail in the attached publication (see appendix).

Tasks 3 and 4 were to create a myometrial cell line that can be tested in-vitro and then in-vivo using xenograft models. These studies are currently underway.

Key Research Accomplishments:

- Created a uterine-specific TSC-2 null mouse
- Demonstrated the presence of myometrial overgrowth and leiomyomas in 100% of uterine-specific TSC-2 null mice.
- Demonstrated upregulation of mTORC1 signaling in these leiomyomas.
- Demonstrated that this mTORC1 signaling is required for myometrial growth in the TSC-2 null mice.
- Demonstrated that estradiol, but not progesterone, is required for myometrial growth in the TSC-2 null mice.
- Demonstrated that the lungs of most older TSC-2 null mice develop myometrial LAM-like tumors.

Reportable Outcomes:

To date, this work has resulted in one publication that was featured on the cover of the September, 2013 issue of Molecular Endocrinology: Prizant et. al. (2013), Molecular Endocrinology 27, 1403-14. Please see appendix.

Conclusion:

To summarize, in the first year of our two-year project we have finished the first half of our proposed studies, creating and characterizing a uterine-specific TSC-2 null mouse. In addition, we have data to support our hypothesis that TSC-2 null LAM lesions can indeed originate in the uterus and metastasize to the lungs. Our future work will focus on isolating myometrial cells from these uteri and creating a cell line that can be used to study signaling and proliferation both in-vitro and in-vivo using xenograft models. We have successfully isolated myometrial cells two times now, and are in the process of passaging these cells to create a stable immortalized cell line. Once we have this cell line, we will proceed with the proposed signaling and xenograft studies.

References:

None.

Uterine-Specific Loss of Tsc2 Leads to Myometrial Tumors in Both the Uterus and Lungs

Hen Prizant, Aritro Sen, Allison Light, Sung-Nam Cho, Francesco J. DeMayo, John P. Lydon, and Stephen R. Hammes

Division of Endocrinology and Metabolism (H.P., A.S., A.L., S.R.H.), Department of Medicine, University of Rochester Medical Center, Rochester, New York 14642; and Department of Molecular and Cellular Biology (S.-N.C., F.J.D.M., J.P.L.), Baylor College of Medicine, Houston, Texas 77030

Lymphangioleiomyomatosis (LAM) is a rare disease characterized by proliferation of abnormal smooth-muscle cells in the lungs, leading to functional loss and sometimes lung transplantation. Although the origin of LAM cells is unknown, several features of LAM provide clues. First, LAM cells contain inactivating mutations in genes encoding Tsc1 or Tsc2, proteins that limit mTORC1 activity. Second, LAM tumors recur after lung transplantation, suggesting a metastatic pathogenesis. Third, LAM is found almost exclusively in women. Finally, LAM shares features with uterine leiomyomas, benign tumors of myometrial cells. From these observations, we proposed that LAM cells might originate from uterine leiomyomas containing Tsc mutations. To test our hypothesis, and to develop mouse models for leiomyoma and LAM, we targeted Tsc2 deletion primarily in uterine cells. In fact, nearly 100% of uteri from uterine-specific Tsc2 knockout mice developed myometrial proliferation and uterine leiomyomas by 12 and 24 weeks, respectively. Myometrial proliferation and mTORC1/S6 activity were abrogated by the mTORC1 inhibitor rapamycin or by elimination of sex steroid production through ovariectomy or aromatase inhibition. In ovariectomized Tsc2 null mice, mTORC1/S6 activity and myometrial growth were restored by estrogen but not progesterone. Thus, even without Tsc2, estrogen appears to be required for myometrial mTORC1/S6 signaling and proliferation. Finally, we found Tsc2 null myometrial tumors in lungs of older Tsc2 uterine-specific knockout females, suggesting that lung LAM-like myometrial lesions may indeed originate from the uterus. This mouse model may improve our understanding of LAM and leiomyomas and might lead to novel therapeutic strategies for both diseases. **(*Molecular Endocrinology* 27: 1403–1414, 2013)**

Pulmonary lymphangioleiomyomatosis (LAM) is a life-threatening, rare disease characterized by proliferation of abnormal smooth muscle cells in the lungs (1–4). With time, patients with LAM develop pulmonary cyst formation, pneumothorax, and progressive pulmonary failure. LAM has 3 unique characteristics: 1) LAM demonstrates strong gender specificity, affecting almost exclusively reproductive-age women; 2) LAM cells express markers of melanoycte differentiation, including gp100 and melanoma-antigen-recognized-by-T-cells (also known as protein melan-A) (5); and 3) LAM cells contain inactivating mutations in tumor suppressor genes Tsc1 or Tsc2. GTPase activating protein function within a protein complex containing Tsc1, Tsc2, and recently discovered TBC1D7 (6), inhibits a small GTPase called Rheb (Ras-homologue-enriched-in-brain), which is a key activator of mammalian target of rapamycin complex 1 (mTORC1). Thus, the Tsc1/Tsc2/TBC1D7 protein complex suppresses mTORC1 signaling pathway and thereby cell growth. In LAM, mutations in the genes encoding either Tsc1 or Tsc2 render this protein complex nonfunctional; therefore, Rheb and downstream mTORC1 are activated, leading to increased cell growth. Interestingly, only 40% of female patients with tuberous sclerosis complex (also with mutations

ISSN Print 0888-8809 ISSN Online 1944-9917
Printed in U.S.A.

Received March 6, 2013. Accepted June 24, 2013.
First Published Online July 2, 2013

Abbreviations: ER, estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin and eosin; LAM, lymphangioleiomyomatosis; mTORC1, mammalian target of rapamycin complex 1; OVX, ovariectomies; PR, progesterone receptor; SMAα, α-smooth muscle actin.

in either the Tsc1 or Tsc2 gene) have lung LAM lesions (7), suggesting that, although Tsc mutations are important in LAM, other "second hits" might be needed for complete development of the disease.

A great mystery in LAM revolves around the origin of the lung LAM cell. Evidence suggests that LAM cells are not derived within the lung, but in fact migrate to the lung from elsewhere in the body through lymphatics or possibly the bloodstream (8–10). For example, many women with LAM who have required lung transplantation develop recurrent LAM tumors in their new lungs that are genetically identical to those in the original lungs (11, 12), suggesting that the new lung LAM cells must have come from elsewhere in the body. Further proof comes from genetic analysis of lung LAM lesions showing that Tsc mutations in lung LAM cells are identical to those found in angiomyolipomas, but not in normal kidney cells, again suggesting an extrapulmonary origin of LAM cells (13–15).

If lung LAM is indeed metastatic, where does it come from? To address this question, one must re-examine the sexual dimorphism of LAM. Notably, LAM progression is greatest in women during their reproductive years (16–18). Furthermore, estradiol appears to be able to promote LAM cell proliferation and possibly metastasis in vitro (19, 20), Together, these observations suggest that estradiol in women might explain the sexual dimorphism of LAM. However, men still produce estradiol. Furthermore, although LAM progression often slows after menopause or ovariectomy (21–23), many anovulatory women still have significant advancement of their disease (24), indicating that estradiol cannot be the only answer.

Another potential explanation for the sexual dimorphism of LAM is the unique presence of a uterus in women. Lung LAM tumors and uterine leiomyomas share many features, including similar-appearing smooth muscle cells, expression of estrogen and progesterone receptors (ERs and PRs), and growth sensitivity to estrogens. Interestingly, a recent study looking for the microscopic presence of LAM cells in the uteri of patients with lung LAM revealed that every patient had leiomyomas, and all but one had actual LAM lesions in the uterus (25). Furthermore, LAM shares features with another rare disease called benign metastasizing leiomyoma, a less aggressive lung disease where it is well established that uterine leiomyomas metastasize to the lungs (26–29). Together, these data suggest that LAM might originate from Tsc2 null myometrial cells, and that estradiol may then promote metastasis and proliferation of these myometrial cells.

To test this hypothesis, and to create a novel mouse model for both leiomyoma and LAM, we generated a primarily uterine-specific Tsc2 knockout mouse by crossing mice containing a floxed Tsc2 gene with mice expressing Cre-recombinase driven by the PR promoter. In fact, in these PR-Cre/Tsc2 null mice, we saw significant loss of Tsc2 expression only in the uterus. Knockout of Tsc2 in the uterus resulted in progressive uterine enlargement and myometrial overgrowth starting at 6 to 12 weeks of age, with leiomyoma formation by 24 weeks of age. Uterine and myometrial growth were prevented by treatment with the mTORC1 inhibitor, rapamycin, or by ovariectomy. Repletion of ovariectomized uterine-specific Tsc2 null mice with estradiol, but not progesterone, restored abnormal uterine growth. Finally, we discovered myometrial Tsc2 null tumors in the lungs of 31-week and older Tsc2 uterine-specific knockout females, suggesting that Tsc2 null myometrial cells, and therefore possibly LAM cells, can indeed migrate from the uterus to the lungs.

Materials and Methods

Animals and sample preparation

Mouse studies were performed in accordance with the guidelines for the Care and Use of Laboratory Animals and were approved by the University Committee on Animal Resources at the University of Rochester. Mice were generated by crossing Tsc2-floxed mice (30), with mice expressing Cre recombinase driven by the PR promoter (31). Tail DNA was isolated for genotyping using appropriate primers (30, 31). Animals were killed between 3 and 36 weeks as indicated. Whole blood was collected via cardiac puncture. Serum was isolated and analyzed for hormone levels at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core. Uteri were removed and weighed, followed by gross dissection and fixation with 10% formalin. For uterine histological analysis sections from paraffin-embedded tissue were stained with either hematoxylin and eosin (H&E) or immunohistochemistry (Immunohistochemistry). The fourth mammary glands were used for whole mount staining using carmine alum (Sigma-Aldrich, St Louis, Missouri). Myometrial thickness was measured in longitudinal uterine sections from at least 3 animals per genotype. Four measurements of the distance between the most outer layer of the myometrium and the endometrium were obtained from each uterine wall and the average of the values was calculated. Lungs were inflated and fixed with 10% formalin.

Western blot

Uteri and lungs were homogenized in RIPA lysis buffer (1×TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, supplemented with PMSF, sodium orthovanadate and protease inhibitor cocktail solutions; Santa Cruz, Santa Cruz, California). Protein lysates were separated on SDS-polyacrylamide gels. Western blots were conducted by using 1:1000 rabbit anti-Tsc2 (Epitomics, Burlingame, California), anti-phospho-S6 (Ser 235/236), anti-S6 and 1:5000 anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling, Danvers, Massachusetts), primary antibodies.

Quantitative real-time PCR

RNA samples were isolated from uterus, ovary, hypothalamus, pituitary, and mammary glands by RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Expression levels of *Tsc2*, *MLANA*, and *GAPDH* mRNA were validated by ΔΔCt method using Taqman primers (Mm00441991_m1, Mm01195780_m1, and Mm03302249_g1, respectively) and a StepOne Plus PCR machine (Applied Biosystems, Foster City, California).

Immunohistochemistry

Uterine sections from paraffin-embedded tissue (5 μm) were deparaffinized and rehydrated in a graded alcohol series. Sections were incubated overnight with 1:1000 anti-smooth-muscle-α-actin (Epitomics) or 1:500 anti-PR (Dako, Glostrup, Denmark), 1:4000 anti-ERα, 1:400 antiphospho S6 (Cell Signaling), 1:300 anti-Ki-67 (Thermo Scientific, Rockford, Illinois), and 1:1000 anti-NKX2.1 (Santa Cruz) antibodies (all from rabbits). On the following day, sections were washed in PBS and incubated with 1:200 antirabbit secondary antibody (5 μL/mL; Vector Laboratories, Burlingame, California) for 1 hour at room temperature. Immunoreactivity was detected using the Vectastain Elite ABC kit (Vector Laboratories). Sections were counterstained by hematoxylin, dehydrated, and mounted using cytoseal 60 (Thermo Scientific).

Rapamycin treatment

Rapamycin (LC Laboratories, Woburn, Massachusetts) was dissolved at 20 mg/mL in ethanol and 1 mg/mL solution was prepared with 5% Tween-80 and 5% polyethylene glycol 400 (Sigma-Aldrich) in PBS. Four-week-old uterine-specific Tsc2 knockout mice and littermate controls were injected with Rapamycin (5 mg/kg, 3 d/wk) ip for 8 weeks. Mice were killed and analyzed as described above.

Letrozole treatment

Letrozole was dissolved in 0.3% hydroxypropyl cellulose (both from Sigma-Aldrich). Four-week-old uterine-specific Tsc2 knockout mice and littermate controls were injected daily with Letrozole (10 μg/mouse/d) sc for 8 weeks. Mice were killed and analyzed as described above.

Ovariectomy and rescue

Four-week-old uterine-specific Tsc2 knockout mice and littermate controls were ovariectomized. Briefly, a dorsal midline incision was made; ovaries were removed, and uterine horns were placed back into the body cavity. The incision was sutured and mice recovered under a heating lamp. Ovariectomized mice were treated with 90-day-release pellet of estradiol (0.5 mg/pellet), progesterone (25 mg/pellet), estradiol + progesterone (Innovative Research of America, Sarasota, Florida), or no pellet for 8 weeks. Mice were killed and analyzed as described above.

Results

Generation of uterine-specific Tsc2 knockout mice

Global deletion of Tsc2 in mice leads to early embryonic lethality (30). Therefore, to study the role of Tsc2 in the mouse uterus, we generated a primarily uterine-specific Tsc2 knockout mouse strain. Mice with floxed Tsc2 ($Tsc2^{flox/flox}$) (30) were crossed with mice expressing Cre recombinase driven by the PR promoter ($PR^{Cre/Cre}$) (31) to generate heterozygous $PR^{Cre/+}/Tsc2^{flox/+}$ mice. These heterozygous mice were then crossed with $Tsc2^{flox/flox}$ females or males, after which $PR^{Cre/+}/Tsc2^{flox/flox}$ (uterine-specific Tsc2 knockout) females were identified by PCR (Figure 1A). Littermates that were heterozygotes or that did not express Cre (referred to as wild-type) were used as controls. In the mouse uterus, low-level PR promoter activity occurs in luminal and glandular epithelium (reflecting less than 20% of total uterine volume) after birth. As mice enter puberty (5–6 wk of age), PR promoter activity increases dramatically in the myometrium (31). Accordingly, uteri of prepubertal 3- to 4-week-old PR-Cre/Tsc2 knockout mice expressed similar levels of *Tsc2* mRNA as heterozygote and wild-type littermates (Figure 1B). In contrast, at 6 (Figure 1C) and 12 (Figure 1D) weeks of age, *Tsc2* mRNA expression in Tsc2 knockout uteri was significantly reduced. As expected, Tsc2 protein expression was essentially undetectable in uteri from these knockout mice (Figure 1, C and D). Notably, *Tsc2* mRNA expression in the pituitary, hypothalamus, and ovary (Figure 1E), as well as the mammary gland (Supplemental Figure 1, published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org), all of which contain low-level PR promoter activity (31), was not significantly reduced in any of the genotypes, demonstrating uterine-specific knockout. In fact, pituitary expression of Tsc2 mRNA may be slightly elevated in the PR-Cre/Tsc2 knockout mouse. Finally, no abnormalities or tumors were seen in whole mounts of PR-Cre/Tsc2 mammary glands (Supplemental Figure 1), again confirming the uterine specificity of our knockout model.

Postpubertal uterine-specific Tsc2 knockout mice develop myometrial proliferation and leiomyomas

No significant morphological changes or differences in uterine weight were observed among the different genotypes in prepubertal mice (Figure 2A). However, starting at 6 weeks of age, uterine-specific Tsc2 knockout mice demonstrated a strong trend toward uterine enlargement, with increased endometrial and myometrial proliferation compared with wild-type and heterozygotic littermates (Figure 2B). At 12 weeks of age, the mTORC1/S6 pathway was constitutively active in Tsc2 knockout uteri, as evidenced by the presence of phosphorylated S6 protein (Figure 2D). Furthermore, uterine size and weight in uterine-specific Tsc2 knockout mice were significantly larger

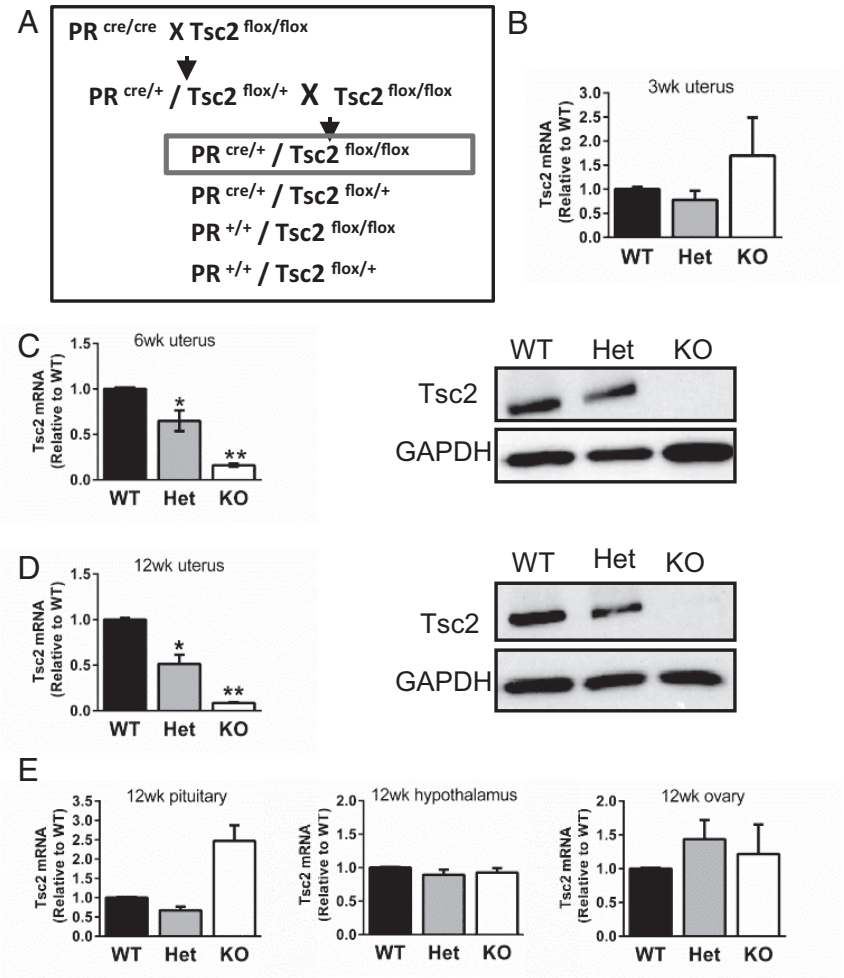

Figure 1. Generation of uterine-specific Tsc2 knockout mice. (A) Schematic of crossing strategy. Uterine *Tsc2* mRNA expression in 3- (B), 6- (C), and 12- (D) week-old wild-type (WT), heterozygotic (Het), and uterine-specific Tsc2 knockout (KO) mice were determined using quantitative real-time PCR. Data were normalized to GAPDH and represented relative to WT uterus. Data are shown as mean ± SEM of at least 3 mice per genotype. *, $P < .05$; **, $P < .0001$ relative to WT by Student *t* test. Uterine Tsc2 protein by immunoblot is shown for 6- (C) and 12- (D) week-old mice. (E) Levels of *Tsc2* mRNA in the pituitary, hypothalamus, and ovary.

compared with heterozygous and wild-type uteri (Figure 2C). Histological analysis demonstrated increased endometrial proliferation, with more endometrial glands and irregular luminal epithelium in uteri of uterine-specific Tsc2 knockout mice (Figure 2C). In addition, histological analysis and measurement of myometrial thickness, along with Ki-67 staining, showed significantly increased myometrial proliferation in uteri of 12-week-old uterine-specific Tsc2 knockout mice relative to wild-type and heterozygotic mice (Figure 2, C, E, and F). In older mice (24 wk of age) Tsc2 knockout uteri were appreciably larger, with continued endometrial overgrowth and internal bleeding (Figure 3A). Notably, for the purposes of this article, we did not focus on the endometrial proliferation, although our results are consistent with PR-Cre-induced phosphate and tensin homolog (PTEN) knockout animals, where PTEN loss leads to enhanced Akt and mTORC1 signaling, resulting in endometrial hyperplasia (32, 33). Focusing on the myometrium, we observed marked myometrial overgrowth, with tumors consistent with leiomyomas (Figure 3A). Although wild-type uteri contained a highly organized, distinct myometrial layer, the Tsc2 knockout myometrium was markedly thickened with rough borders and irregular, disoriented cells that stained for α-smooth muscle actin (SMAα) (Figure 3, B–G). In addition, Tsc2 knockout myometrium expressed high levels of phosphorylated S6 protein relative to wild-type myometrium, reflecting Tsc2 loss and mTORC1 activation (Figure 3, H and I). Notably, similar to leiomyomas, uteri from uterine-specific Tsc2 knockout mice expressed ERα and PR (Figure 3, J and K), suggesting that ER and/or PR signaling might play a role in the abnormal myometrial growth. In addition, although *MLANA* mRNA (encodes the melanocytic protein melanoma-antigen-recognized-by-T-cells—a protein known to be upregulated in LAM) was barely detected in the uteri of 12-week-old wild-type mice, *MLANA* mRNA was markedly elevated in uteri of uterine-specific Tsc2 knockout mice (Figure 3L), confirming yet another similarity between the Tsc2 null uteri and LAM cells. Last, markedly elevated myometrial proliferation was corroborated by Ki-67 staining at 24 weeks of age (Figure 3M).

Rapamycin prevents abnormal growth of Tsc2 knockout uteri

Rapamycin inhibits mTORC1 and is used as an immunosuppressant to prevent rejection after transplantation. Rapamycin effectively blocks tumor development in the global Tsc2+/− mouse model (34) and reduces tumor size in Eker rats (35). Four-week-old (prepubertal) uterine-specific Tsc2 knockout, heterozygotic, and wild-type mice were treated with rapamycin for 8 weeks. Rapamycin treatment completely prevented the abnormal growth seen in 12-week-old uterine-specific Tsc2 knockout mice, with no differences in morphology, size, and weight relative to wild-type mice (Figure 4, A and B). Confirming that rapamycin effectively inhibited mTORC1, and S6

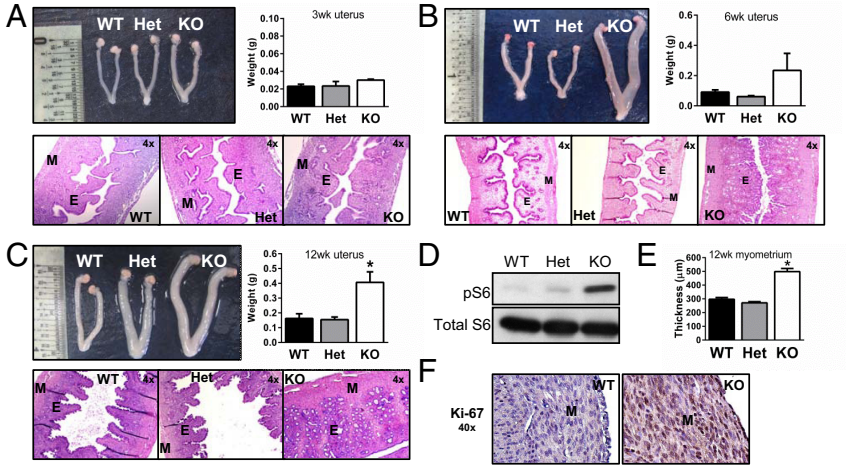

Figure 2. Uteri of uterine-specific Tsc2 KO mice show abnormal growth after puberty. (A) No detectable differences were seen in size (upper left panel), weight (upper right panel), or morphology (lower panel) between uteri of prepubertal 3-week-old WT, Het, and uterine-specific Tsc2 KO mice. At 6 (B) and 12 (C) weeks of age, Tsc2 KO uteri were larger (upper left panels) and heavier (upper right panels), and H&E stains showed increased endometrial (E) and myometrial (M) proliferation with respect to Het and WT uteri (lower panels). (D) Immunoblot demonstrating phosphorylated and total S6 in uteri of 12-week-old WT, Het, or KO mice. (E) Average myometrial thickness of 12-week-old WT, Het, or KO mice. (F) Uterine sections from 12-week-old WT and KO mice stained for Ki-67. Photos are representative of at least 3 mice per genotype. Results portray the mean ± SEM of at least 4 mice per genotype. *, $P < .05$ relative to WT by Student *t* test.

phosphorylation was low in Tsc2 knockout uteri (as opposed to constitutive S6 phosphorylation in untreated Tsc2 knockout uteri) (Figure 4C). Thus, as expected, mTORC1 activity appears critical for the myometrial overgrowth in uterine-specific Tsc2 knockout mice.

Ovariectomy prevents abnormal growth of Tsc2 knockout uteri and estrogen stimulates myometrial proliferation

Uterine leiomyomas and LAM usually develop during the reproductive years in women. In contrast, men with Tsc mutations rarely develop LAM (3). Clinical and biochemical studies report an important role for estrogen, and possibly progesterone, in the promotion of leiomyoma growth (36). Furthermore, like leiomyomas, lung LAM cells express ERs and PRs (2, 37) and appear to proliferate in response to estradiol (38). Because myometrial tumors from Tsc2 knockout uteri express PR and ERα (Figure 3, J and K), we examined the importance of steroid signaling in the abnormal uterine growth of uterine-specific Tsc2 knockout mice. Ovariectomies (OVX) were performed in 4-week-old uterine-specific Tsc2 knockout and wild-type mice. After 8 more weeks, uteri were examined. In the absence of ovarian steroids, uteri from uterine-specific Tsc2 knockout mice looked identical to those from wild-type mice, with no evidence of myometrial or endometrial proliferation by gross examination and weight (Figure 5A). Thus, ovarian steroids appear necessary for abnormal uterine growth. To determine which ovarian steroid regulated myometrial growth in uterine-specific Tsc2 knockout mice, pellets containing estradiol (5.5 μg/d/mouse), progesterone (280 μg/d/mouse), or both, were applied to OVX mice. Mice were treated for 8 weeks, after which they needed to be killed due to known estradiol-implant effects on the bladder that lead to urinary retention (39). After 8 weeks of steroid treatment, excess myometrial growth was restored to OVX uterine-specific

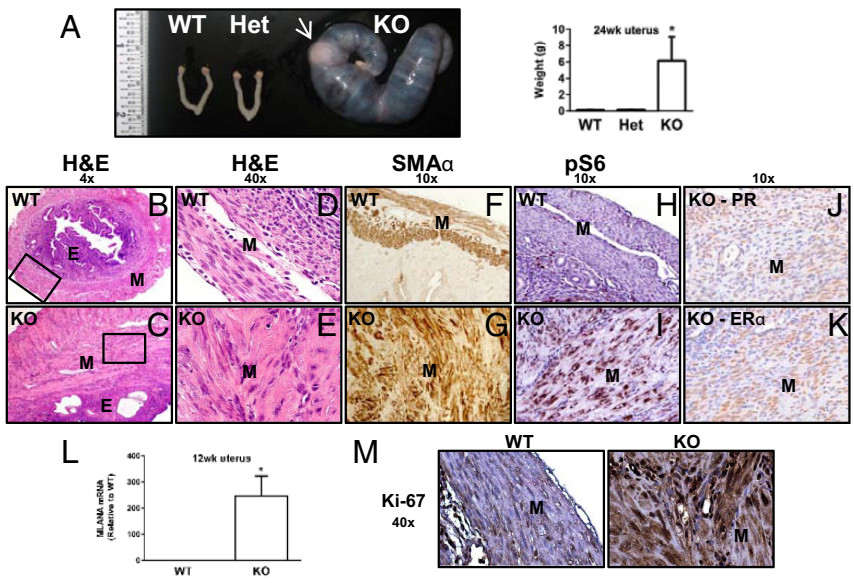

Figure 3. Uteri from 24-week-old uterine-specific Tsc2 KO mice contain leiomyomas and share features with LAM. (A) Uteri of 24-week-old WT, Het, and uterine-specific Tsc2 KO mice. Tsc2 KO uteri were larger, often blood-filled, and contained leiomyomas (arrow). (B–K) Uterine sections from 24-week-old WT and KO mice stained with H&E (B–E; M, myometrium, E, endometrium), anti-SMAα (F, G), antiphosphorylated S6 (H, I), anti-PR (J), and anti-ERα (K). (L) Uterine *MLANA* mRNA expression in 12-week-old WT and KO mice were determined using quantitative real-time PCR. Data were normalized to GAPDH and represented relative to WT uterus. Data are shown as mean ± SEM of at least 3 mice per genotype. (M) Uterine sections from 24-week-old WT and KO mice stained for Ki-67. Photos are representative of at least 3 mice per genotype. *, $P < .05$ relative to WY by Student *t* test.

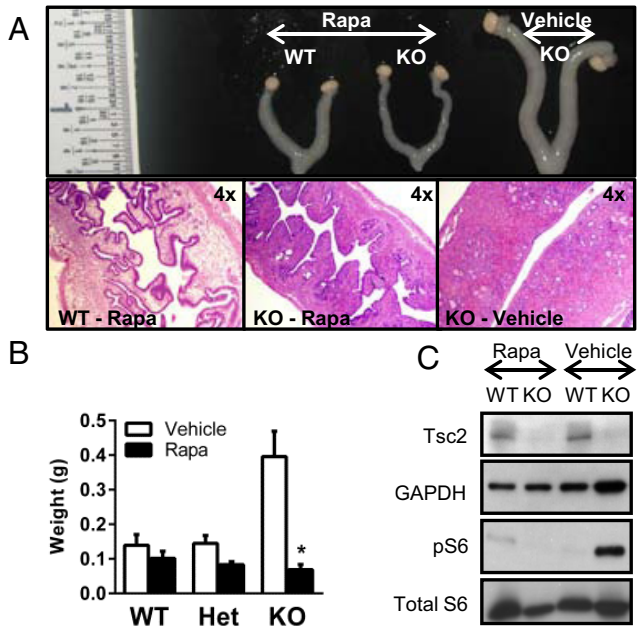

Figure 4. Rapamycin prevents abnormal growth of Tsc2 KO uteri. Four-week-old WT, Het, and uterine-specific Tsc2 KO mice were injected with rapamycin (Rapa, 5 mg/kg ip, 3 d/wk for 8 wk). (A) At 12 weeks of age, rapamycin prevented uterine overgrowth of KO mice as seen by size (upper panel), morphology (lower panel), and (B) weight. Photos are representative from 3 experiments with identical results. Results portray the mean ± SEM of 3 mice per group per treatment. *, $P < .05$ relative to untreated uterine-specific Tsc2 KO by Student *t* test. (C) To confirm inhibition of mTORC1, levels of uterine Tsc2, phosphorylated S6, total S6, and GAPDH were measured by immunoblot.

Tsc2 knockout mice by estradiol alone, as evidenced by uterine size and weight, as well as by thickened myometrial growth (Figure 5A). Uterine size and weight were also increased in wild-type OVX mice exposed to estradiol; however, these increases were primarily due to increased endometrial proliferation, with normal myometrial architecture (Figure 5A). In contrast, progesterone alone had no significant effect on uterine morphology or weight in either wild-type or uterine-specific Tsc2 knockout OVX mice, suggesting that progesterone is not a major regulator of myometrial growth in the absence of Tsc2 expression. Finally, treatment of OVX wild-type mice with estradiol plus progesterone decreased proliferation of epithelial and glandular tissue, reflected by the slightly decreased size and uterine weight relative to OVX wild-type treated with E2 alone (Figure 5A). This observation is consistent with progesterone's known opposing effects on estradiol-mediated endometrial proliferation (40). However, combined estradiol and progesterone treatment of OVX uterine-specific Tsc2 knockout mice increased uterine size and weight, with continued endometrial and myometrial growth (and often unilateral fluid buildup in one uterine horn, although this observation was variable), suggesting that, in the setting of Tsc2 loss, progesterone does not as effectively oppose estrogen action in the uterus. Intriguingly, although Tsc2 expression remained low in the uteri of OVX uterine-specific Tsc2 knockout mice, S6 phosphorylation was no longer significant (Figure 5B), suggesting that mTORC1 activity might require steroid signaling, even in the absence of Tsc2. In fact, estradiol, but not progesterone, treatment of OVX uterine-specific Tsc2 knockout mice rescued mTORC1 activity, as evidenced by the statistically significant increase in total uterine S6 phosphorylation by Western blot (Figure 5B), and an increase in myometrial S6 phosphorylation by immunohistochemistry (Figure 5C). Similarly, estradiol plus progesterone enhanced myometrial S6 phosphorylation by immunostochemistry (Figure 5C) and total uterine S6 phosphorylation by Western blot (Figure 5B), although the latter was not quite statistically significant in this series of 3 uteri.

Aromatase inhibition reduces myometrial proliferation in Tsc2 knockout uteri

Estrogens are generated from androgens by aromatase. Aromatase inhibitors, such as letrozole, are used in breast cancer patients to block estrogen production and subsequent ER signaling. Because estradiol regulated myometrial growth in our mouse model, we predicted that aromatase inhibition would attenuate growth in 12-week-old uterine-specific Tsc2 knockout mice. Starting at 4 weeks, uterine-specific Tsc2 knockout and wild-type mice were treated with letrozole for 8 weeks. As expected, uterine size and weight were significantly lower in letrozole-treated vs untreated uterine-specific Tsc2 knockout mice (Figure 6, A and B), confirming that estradiol is required for proliferation in the absence of Tsc2. Importantly, S6 phosphorylation in the uteri of letrozole-treated uterine-specific Tsc2 knockout was markedly reduced relative to intact uterine-specific Tsc2 knockout mice, with levels no different than wild-type uteri (Figure 6D), again suggesting that estradiol is required for mTORC1 activity, even in the absence of Tsc2. Finally, although estradiol levels were relatively low in all mice examined, serum LH levels were elevated in letrozole-treated wild-type and knockout mice (Figure 6C), confirming that chronic estradiol production was reduced by letrozole.

Metastatic myometrial lesions in the lungs of uterine-specific Tsc2 knockout mice

As mentioned, this mouse model was created to determine whether myometrial, or possibly LAM, lung lesions could be derived from the uterus. Interestingly, we found that most uterine-specific Tsc2 knockout females that

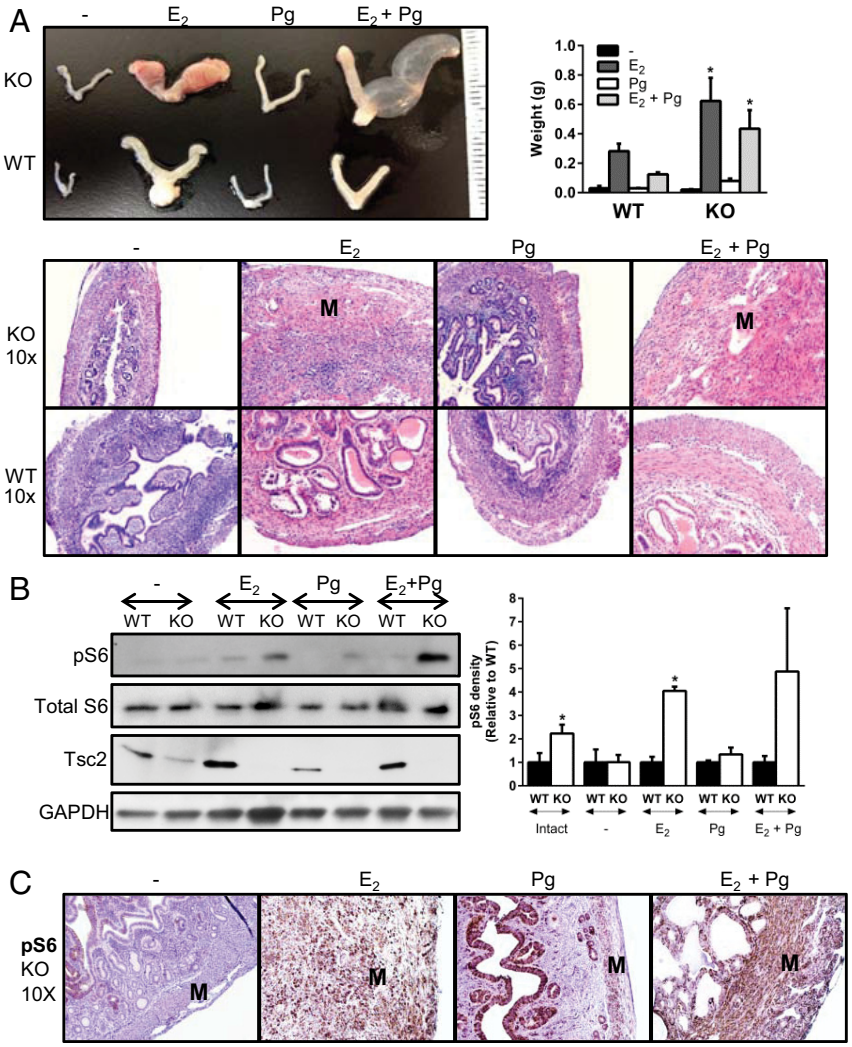

Figure 5. Ovariectomy prevents abnormal growth of Tsc2 KO uteri. Four-week-old WT and uterine-specific Tsc2 KO mice were OVX and treated with pellets of estradiol (E_2, 0.5 mg/pellet), progesterone (Pg, 25 mg/pellet), both, or neither for 8 weeks. (A) At 12 weeks of age, uterine gross morphology (upper left panel), weight (upper right panel), and microscopic morphology (lower panel) were analyzed. M, myometrium. Results portray the mean ± SEM of at least 3 mice per group per treatment. *, $P < .05$ relative to untreated OVX uterine-specific Tsc2 KO by Student *t* test. (B) Levels of Tsc2, GAPDH, and phosphorylated and total S6 were examined by immunoblot in OVX WT and KO uteri (left). Treatments are indicated. Quantification by densitometry of phosphorylated S6/total S6 is shown on the right. The *y*-axis is pS6/tS6 of KO relative to WT uteri for each condition. Intact represents mice that never received OVX. *, $P < .05$ relative to WT pairs by Student *t* test, n ≥ 3 for each condition. (C) Immunohistochemistry for phosphorylated S6 was performed using sections from the indicated Tsc2 KO uteri.

reached 31 weeks and up (6 of 8) contained myometrial tumors in the lungs (Figure 7A). In contrast, no mice age 24 weeks or lower had any detectable lung tumors. Western blot analysis of uterine and lung tumors from a representative 34-week-old Tsc2 knockout mouse demonstrated extremely low Tsc2 protein expression (Figure 7B). Tsc2 expression was normal in unaffected lungs of these mice. The very faint Tsc2 protein expression in the KO lanes most likely reflects the heterogeneity of the tumors and the difficulty in specifically manually dissecting only tumor tissue. Moreover, the lung tumors expressed PR, ERα, SMAα, and phosphorylated-S6 positive (all seen in the uterine myometrial lesions), but not the lung marker, Nkx2.1 (Figure 7C). Finally, when examining lungs in the knockout animals for early tumor formation, we find tumors originating from the lymphatics, as evidenced by LYVE1-positive staining of lymphatic endothelial cells surrounding the tumor (Supplemental Figure 2). These data strongly suggest that the lung tumors were metastatic from the uterus, perhaps entering the lungs via the lymphatics (as is seen in LAM), confirming that LAM-like myometrial lung tumors could indeed be derived from uterus.

Discussion

Although progress has been made in diagnosing and treating LAM, a cure remains elusive. One difficulty in finding a cure is that both the origin of LAM cells and the mechanism of the sexual dimorphism in LAM are still not well understood. Furthermore, because of the lack of amenable animal models, the relative importance of mTORC1 vs estrogen signaling in LAM is not well characterized. Here we started with the hypothesis that lung LAM cells, which resemble uterine leiomyoma cells, might be derived from uterine myometrial cells containing mutations in 1 of the 2 TSC alleles. We further proposed that the combination of estrogen and de-repressed mTORC1 signaling might be required for LAM proliferation. To test our hypothesis, we specifically knocked out Tsc2 in uterine cells. Nearly 100% of the uterine-specific Tsc2 knockout mice developed myometrial thickening by 12 weeks of age and uterine leiomyomas by 24 weeks of age. These uterine tumors share many features of leiomyoma and LAM, including PR, ERα, SMAα, and phosphorylated S6 protein expression. These uteri also express elevated *MLANA* mRNA levels, a melanocyte differentiation marker commonly seen in LAM cells. Finally, 75% of mice older than 31

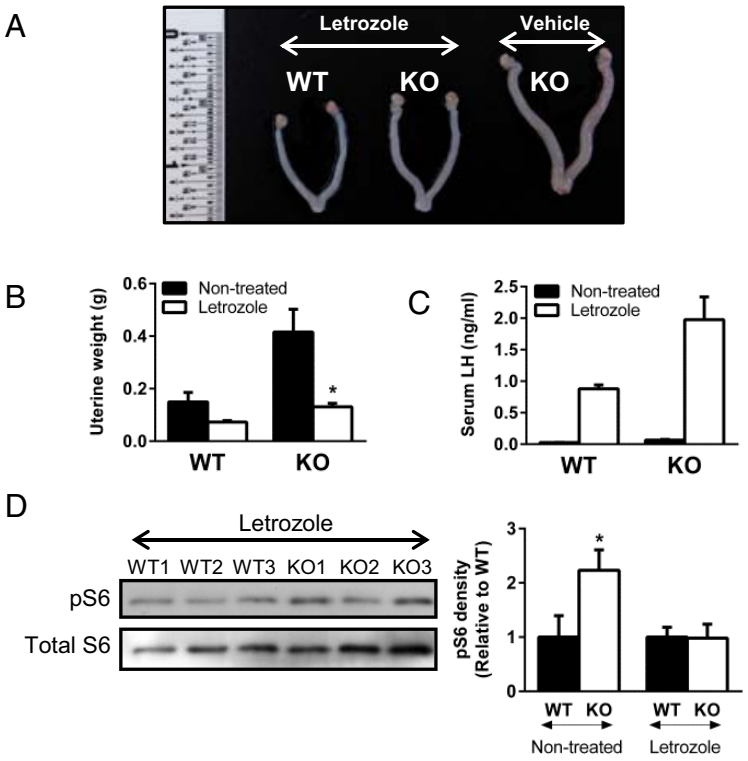

Figure 6. Letrozole prevents abnormal growth of Tsc2 KO uteri. Four-week-old WT and uterine-specific Tsc2 KO mice were injected with letrozole (10 μg/mouse daily) sc for 8 weeks. Uterine gross morphology (A) and weights (B) in 12-week-old KO treated with letrozole are similar to those of WT. (C) LH levels in serum were analyzed by sandwich immunoradiometric assay. Results portray the mean ± SEM of 3 mice per treatment. *, $P < .05$ relative to untreated uterine-specific Tsc2 KO by Student *t* test. (D) Levels of phosphorylated and total S6 were examined by immunoblot, with 3 representative samples from letrozole-treated WT and KO mice shown on the left. Quantification by densitometry of phosphorylated S6/total S6 for WT and KO mice both treated and not treated with letrozole is shown on the right. The *y*-axis is pS6/tS6 of letrozole-treated vs untreated uteri for WT and KO mice. *, $P < .05$ relative to untreated WT by Student *t* test, n ≥ 3 for each condition.

weeks of age developed lung myometrial lesions. Although the global lung pathology is not identical to LAM, the individual tumors share some pathologic features similar to LAM, including PR, ERα, SMAα, and phosphorylated-S6 protein expression. Thus, this mouse strain may serve as a useful model for both leiomyoma and LAM.

The first key physiologic feature shared between Tsc2 null mouse myometrial cells and LAM is the dependence on mTORC1 signaling for proliferation. As seen in LAM and other models (41, 42), rapamycin completely blocked myometrial overgrowth in our model (Figure 4), even in the presence of ovarian steroids. This observation demonstrates that mTORC1 signaling is necessary for the proliferation of Tsc2-null mouse myometrial cells. Similarly, LAM progression appears to require mTORC1 signaling, as rapamycin analogs stabilize lung function and reduce symptoms in LAM patients (41, 43, 44). Importantly, however, lung function again declines after discontinuation of therapy (45–47), suggesting that mTORC1 inhibition only slows growth but does not destroy LAM cells. Thus, the need for additional therapeutic targets in LAM remains.

Another potential therapeutic target, and the second feature shared between Tsc2 null myometrial cells and LAM cells, is the dependence on estrogen. We found that elimination of steroid signaling by ovariectomy abrogated myometrial overgrowth in uterine-specific Tsc2 knockout mice (Figure 5). This result demonstrates the critical role of steroid signaling for proliferation, even in the absence of Tsc2. Importantly, although elimination of ovarian steroidogenesis blocked myometrial proliferation in Tsc2 knockout uteri, growth was restored by the addition of estradiol but not progesterone (Figure 5A). This observation confirms that estradiol is the major ovarian steroid regulating myometrial cell proliferation and is consistent with previous work supporting the role of estrogen in the promotion of both leiomyoma and LAM. These earlier studies demonstrated that LAM and leiomyoma cells expressed ERα and progressed most rapidly in women during pregnancy and while taking oral contraceptives, when estrogen levels are highest (48–54). Furthermore, in vitro studies demonstrated estrogen-induced increased proliferation of primary human LAM cells as well as ELT3 Tsc2-null Eker rat leiomyoma cells (48, 55). In fact, estradiol not only regulated ELT3 cell proliferation, but also promoted metastatic spread of ELT3 cells in xenograft models (19). Notably, ERα promotes cell growth via both genomic transcriptional responses as well as nongenomic cross-talk between ERα and growth factor receptors (56). Thus, inhibition of genomic and nongenomic ERα signaling might have greater potential in battling LAM than blockade of any one individual pathway. Importantly, we found that ovariectomized or letrozole-treated uterine-specific Tsc2 knockout mice demonstrated markedly reduced uterine/myometrial mTORC1 signaling relative to untreated knockout mice, as reflected by decreased phosphoylration of S6 (Figure 5, B and C, Figure 6D). Furthermore, estradiol or estradiol/progesterone treatment of ovariectomized uterine-specific Tsc2 knockout mice rescued mTORC1 signaling in the myometrium. Together, these observations suggest that estradiol is required for complete activation of mTORC1/S6 signaling in myometrial cells, even in the absence of Tsc2 expression, underscoring the importance of estradiol for myometrial and possibly LAM proliferation. In fact, the

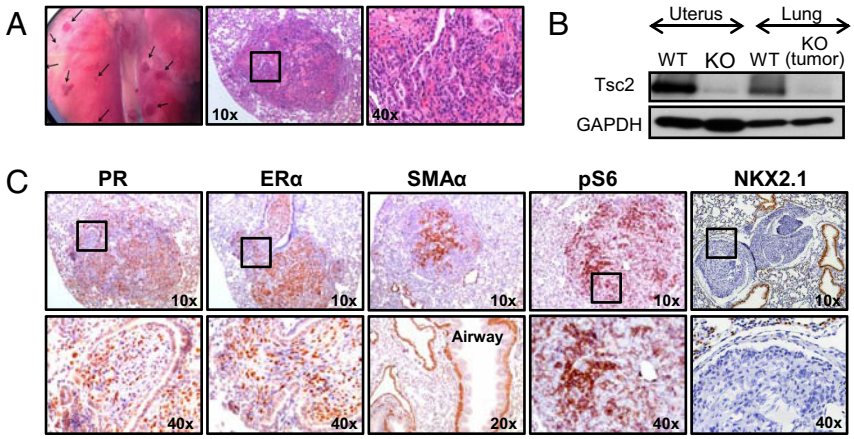

Figure 7. Myometrial tumors in the lungs of uterine-specific Tsc2 KO mice. (A) Lung tumors (arrows) were found in 75% (6/8) of 31 week and older uterine-specific Tsc2 KO mice. H&E staining showed leiomyoma-like appearance. (B) Immunoblots of uterine and a lung tumor from a representative mouse demonstrated lack of Tsc2 protein. (C) Immunohistochemical staining demonstrated that the lung tumors were PR, ERα, SMAα, and phosphorylated S6 positive, but negative for the lung marker Nkx2.1.

need for estradiol signaling to keep mTORC1 signaling elevated in cells lacking Tsc2 suggests that anti-estrogen treatment might be an effective means of suppressing proliferative signals in LAM.

An additional steroid that might contribute to the gender specificity of LAM is progesterone. The role of progesterone in leiomyomas and LAM is not well understood. Some studies have shown that progestins might promote leiomyoma growth and survival and therefore might be contraindicated in patients with leiomyomas or LAM (57, 58). Accordingly, although early studies suggested that progestins might improve symptoms in LAM patients (59, 60), a later study demonstrated no difference and possibly deterioration of LAM symptoms on treatment with progestin (21). In our model, progesterone did not promote myometrial proliferation on its own. Furthermore, the full opposing effect of progesterone on estradiol-mediated effects was not present in Tsc2 knockout uteri, as myometrial cells proliferated equally well in response to estradiol alone or estradiol plus progesterone (Figure 5). Thus, progesterone does not appear to be a major regulator of Tsc2 null myometrial growth.

The final feature shared between our uterine-specific Tsc2 knockout mouse and LAM is the presence of lung tumors. Genetic data suggest that LAM might originate from outside the lungs (8). Moreover, case studies have described recurrence of LAM within lungs after lung transplantation, again suggesting a metastatic origin of LAM (12, 61). Because leiomyoma cells share many features with LAM cells, and because leiomyomas are found exclusively in women, the uterus is an intriguing potential source of LAM cells. Consistent with this possibility, a recent study found uterine leiomyomas in every LAM patient studied, with microscopic uterine LAM lesions in all but one patient (25). Furthermore, in the less aggressive lung disease, benign metastasizing leiomyoma, myometrial cells metastasize to the lungs (62, 63). We therefore propose that loss of Tsc2 in myometrial cells might result in a more aggressive form of benign metastasizing leiomyoma that, in the presence of estradiol, leads to proliferating myometrial tumors and possibly LAM. In fact, in 75% of mice that survived to 31 weeks of age, we found myometrial lung tumors that were Tsc2 and Nkx2.1 negative, but expressed pS6, PR, ERα, and SMAα (Figure 7). Furthermore, some of these tumors can be seen emerging from the lymphatics (Supplemental Figure 2), which is thought to be a common mode of lung entry for LAM cells. All of these data are consistent with metastatic leiomyoma as well as LAM and suggest that uterine myometrial Tsc2 null cells can indeed travel to the lungs. Although we do not see significant loss of Tsc2 expression in any other tissue, nor do we see tumors in tissues other than the uterus and lungs (and the lung tumors are present less frequently and only later in life), it is still possible that the lung lesions described here were derived from other small pockets of PR-positive tissues, or even from PR-positive smooth muscle cells within the lungs. To address this possibility, studies are currently being performed where uteri (but not ovaries) are removed from uterine-specific Tsc2 knockout mice before puberty and lungs are examined at 31 weeks.

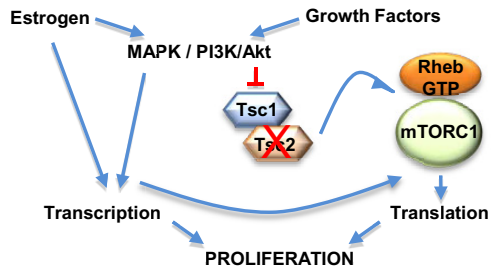

Figure 8. Schematic model for potential estrogen/mTORC1 crosstalk in LAM. Estrogen promotes proliferation both transcriptionally and through nongenomic MAPK/Akt signaling. This nongenomic kinase signaling can both modulate ER-mediated transcription and regulate mTORC1 signaling through both Tsc1/2-dependent and possibly -independent mechanisms. This complex of mTORC1 inhibitor proteins likely includes TBC1D7, as well as Tsc1 and Tsc2. mTORC1 regulates proliferation through many pathways, including enhancement of translation.

Of note, alternative rodent models for LAM have provided important information. Perhaps the best known is the Eker rat, which contains a heterozygotic inactivating mutation in the Tsc2 locus. Eker rats develop tumors in multiple locations within the body, including the uterine myometrium (64). In fact, this model was the first to demonstrate that Tsc2 deletion in the myometrium can lead to leiomyoma formation. However, Eker rats did not develop myometrial lung lesions. Furthermore, because leiomyoma formation requires inactivation of the second Tsc2 allele, only 65% of females develop leiomyomas, and the time to leiomyoma formation is over 1 year. In our uterine-specific Tsc2 knockout mouse model, myometrial cell hyperproliferation occurs in nearly 100% of the mice by 12 weeks and leiomyoma formation by 24 weeks, potentially making this model more amenable for studying leiomyoma and LAM. In addition to the rats, both Tsc1 and Tsc2 global knockout mice have been made (65–68). Complete knockout mice are embryonically lethal, but heterozygotes develop tumors that, as in Eker rats, contain inactivation of the second allele. However, unlike the model presented here, none of these mice develop uterine leiomyomas or lung myometrial lesions. Using an alternative approach, labeled Tsc2 null angiomyolipoma cells injected into mice intratracheally developed histopathologic changes within the lung that were consistent with LAM as well as dissemination of Tsc2 null cells throughout the lymphatics, thus creating a novel model for examining LAM progression (69). In addition, a recent mouse model has been introduced whereby Tsc2 null mouse kidney cells were injected into the flanks of nude mice; flank tumors were removed and grown in vitro, and cultured cells were injected into the tail veins of nude mice (70). Injected nude mice then developed lung lesions consistent with pulmonary LAM. Like the intratracheal system, this powerful model holds great promise, but both involve using immunocompromised mice and do not address the potential uterine origin of the LAM cell.

In summary, mice with conditional knockout of Tsc2 in the uterus provide a new model for both leiomyoma and possibly LAM. Myometrial tumors in the uteri and lungs share a number of key features with LAM, and both estrogen and mTORC1 signaling are necessary for growth (Figure 8). Furthermore, although not completely identical to overall LAM lung pathology (for example, the lungs in these mice did not develop the cysts seen in LAM patients), the presence of myometrial tumors that share many features of LAM within the lungs of uterine-specific Tsc2 knockout mice suggests that LAM cells might metastasize from the uterus. This novel model may prove useful in understanding the pathophysiology of leiomyoma and LAM, identifying cellular events that allow LAM cells to proliferate and travel to the lungs, and developing novel therapeutic strategies for the treatment of LAM.

Acknowledgments

We thank Diego Castrillon (UT Southwestern) for his invaluable advice. This work was funded by grants from the LAM foundation and the DOD (TS110032). The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core is supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development/National Institutes of Health (SCCPIR) Grant U54-HD28934.

Address all correspondence and requests for reprints to: Stephen R. Hammes, MD, PhD, Division of Endocrinology and Metabolism, University of Rochester School of Medicine, 601 Elmwood Avenue, Rochester, NY 14642. E-mail: Stephen_hammes@urmc.rochester.edu.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Zhe X, Schuger L.** Combined smooth muscle and melanocytic differentiation in lymphangioleiomyomatosis. *J Histochem Cytochem*. 2004;52(12):1537–1542.
2. **Ferrans VJ, Yu ZX, Nelson WK, et al.** Lymphangioleiomyomatosis (LAM): a review of clinical and morphological features. *J Nihon Med Sch*. 2000;67(5):311–329.
3. **Taveira-DaSilva AM, Pacheco-Rodriguez G, Moss J.** The natural history of lymphangioleiomyomatosis: markers of severity, rate of progression and prognosis. *Lymphat Res Biol*. 2010;8(1):9–19.
4. **Henske EP, McCormack FX.** Lymphangioleiomyomatosis—a wolf in sheep's clothing. *J Clin Invest*. 2012;122(11):3807–3816.
5. **Klarquist J, Barfuss A, Kandala S, et al.** Melanoma-associated antigen expression in lymphangioleiomyomatosis renders tumor cells susceptible to cytotoxic T cells. *Am J Pathol*. 2009;175(6):2463–2472.
6. **Dibble CC, Elis W, Menon S, et al.** TBC1D7 is a third subunit of the TSC1-TSC2 complex upstream of mTORC1. *Mol Cell*. 2012;47(4):535–546.
7. **McCormack F, Brody A, Meyer C, et al.** Pulmonary cysts consistent with lymphangioleiomyomatosis are common in women with tuberous sclerosis: genetic and radiographic analysis. *Chest*. 2002;121(suppl 3):61S.
8. **Yu J, Henske EP.** mTOR activation, lymphangiogenesis, and estrogen-mediated cell survival: the "perfect storm" of pro-metastatic factors in LAM pathogenesis. *Lymphat Res Biol*. 2010;8(1):43–49.
9. **Seyama K, Kumasaka T, Kurihara M, Mitani K, Sato T.** Lymphangioleiomyomatosis: a disease involving the lymphatic system. *Lymphat Res Biol*. 2010;8(1):21–31.
10. **Glasgow CG, Taveira-Dasilva AM, Darling TN, Moss J.** Lymphatic involvement in lymphangioleiomyomatosis. *Ann N Y Acad Sci*. 2008;1131:206–214.
11. **Astrinidis A, Khare L, Carsillo T, et al.** Mutational analysis of the tuberous sclerosis gene TSC2 in patients with pulmonary lymphangioleiomyomatosis. *J Med Genet*. 2000;37(1):55–57.
12. **Karbowniczek M, Astrinidis A, Balsara BR, et al.** Recurrent lymphangiomyomatosis after transplantation: genetic analyses reveal a

metastatic mechanism. *Am J Respir Crit Care Med*. 2003;167(7):976–982.
13. **Smolarek TA, Wessner LL, McCormack FX, Mylet JC, Menon AG, Henske EP.** Evidence that lymphangiomyomatosis is caused by TSC2 mutations: chromosome 16p13 loss of heterozygosity in angiomyolipomas and lymph nodes from women with lymphangiomyomatosis. *Am J Hum Genet*. 1998;62(4):810–815.
14. **Carsillo T, Astrinidis A, Henske EP.** Mutations in the tuberous sclerosis complex gene TSC2 are a cause of sporadic pulmonary lymphangioleiomyomatosis. *Proc Natl Acad Sci USA*. 2000;97(11):6085–6090.
15. **Sato T, Seyama K, Fujii H, Maruyama H, et al.** Mutation analysis of the TSC1 and TSC2 genes in Japanese patients with pulmonary lymphangioleiomyomatosis. *J Hum Genet*. 2002;47(1):20–28.
16. **Oh YM, Mo EK, Jang SH, et al.** Pulmonary lymphangioleiomyomatosis in Korea. *Thorax*. 1999;54(7):618–621.
17. **Johnson SR, Tattersfield AE.** Clinical experience of lymphangioleiomyomatosis in the UK. *Thorax*. 2000;55(12):1052–1057.
18. **Kitaichi M, Nishimura K, Itoh H, Izumi T.** Pulmonary lymphangioleiomyomatosis: a report of 46 patients including a clinicopathologic study of prognostic factors. *Am J Respir Crit Care Med*. 1995;151(2, Pt 1):527–533.
19. **Yu JJ, Robb VA, Morrison TA, et al.** Estrogen promotes the survival and pulmonary metastasis of tuberin-null cells. *Proc Natl Acad Sci USA*. 2009;106(8):2635–2640.
20. **Glassberg MK, Elliot SJ, Fritz J, et al.** Activation of the estrogen receptor contributes to the progression of pulmonary lymphangioleiomyomatosis via matrix metalloproteinase-induced cell invasiveness. *J Clin Endocrinol Metab*. 2008;93(5):1625–1633.
21. **Taveira-DaSilva AM, Stylianou MP, Hedin CJ, Hathaway O, Moss J.** Decline in lung function in patients with lymphangioleiomyomatosis treated with or without progesterone. *Chest*. 2004;126(6):1867–1874.
22. **Johnson SR, Tattersfield AE.** Decline in lung function in lymphangioleiomyomatosis: relation to menopause and progesterone treatment. *Am J Respir Crit Care Med*. 1999;160(2):628–633.
23. **Adamson D, Heinrichs WL, Raybin DM, Raffin TA.** Successful treatment of pulmonary lymphangiomyomatosis with oophorectomy and progesterone. *Am Rev Respir Dis*. 1985;132(4):916–921.
24. **Khalife WI, Mahmoud F, Larson E, Hardie R.** Pulmonary lymphangioleiomyomatosis in a postmenopausal woman: case report with review of literature. *S D J Med*. 2005;58(4):139–143.
25. **Hayashi T, Kumasaka T, Mitani K, et al.** Prevalence of uterine and adnexal involvement in pulmonary lymphangioleiomyomatosis: a clinicopathologic study of 10 patients. *Am J Surg Pathol*. 2011;35(12):1776–1785.
26. **Tietze L, Günther K, Hörbe A, et al.** Benign metastasizing leiomyoma: a cytogenetically balanced but clonal disease. *Hum Pathol*. 2000;31(1):126–128.
27. **Bowen JM, Cates JM, Kash S, et al.** Genomic imbalances in benign metastasizing leiomyoma: characterization by conventional karyotypic, fluorescence in situ hybridization, and whole genome SNP array analysis. *Cancer Genet*. 2012;205(5):249–254.
28. **Goyle KK, Moore DF Jr, Garrett C, Goyle V.** Benign metastasizing leiomyomatosis: case report and review. *Am J Clin Oncol*. 2003;26(5):473–476.
29. **Awonuga AO, Shavell VI, Imudia AN, Rotas M, Diamond MP, Puscheck EE.** Pathogenesis of benign metastasizing leiomyoma: a review. *Obstet Gynecol Surv*. 2010;65(3):189–195.
30. **Hernandez O, Way S, McKenna J 3rd, Gambello MJ.** Generation of a conditional disruption of the Tsc2 gene. *Genesis*. 2007;45(2):101–106.
31. **Soyal SM, Mukherjee A, Lee KY, et al.** Cre-mediated recombination in cell lineages that express the progesterone receptor. *Genesis*. 2005;41(2):58–66.
32. **Daikoku T, Hirota Y, Tranguch S, et al.** Conditional loss of uterine Pten unfailingly and rapidly induces endometrial cancer in mice. *Cancer Res*. 2008;68(14):5619–5627.
33. **Kim TH, Franco HL, Jung SY, et al.** The synergistic effect of Mig-6 and Pten ablation on endometrial cancer development and progression. *Oncogene*. 2010;29(26):3770–3780.
34. **Lee L, Sudentas P, Donohue B, et al.** Efficacy of a rapamycin analog (CCI-779) and IFN-gamma in tuberous sclerosis mouse models. *Genes Chromosomes Cancer*. 2005;42(3):213–227.
35. **Kenerson H, Dundon TA, Yeung RS.** Effects of rapamycin in the Eker rat model of tuberous sclerosis complex. *Pediatr Res*. 2005;57(1):67–75.
36. **Maruo T, Ohara N, Wang J, Matsuo H.** Sex steroidal regulation of uterine leiomyoma growth and apoptosis. *Hum Reprod Update*. 2004;10(3):207–220.
37. **Benassayag C, Leroy MJ, Rigourd V, et al.** Estrogen receptors (ERα/ERβ) in normal and pathological growth of the human myometrium: pregnancy and leiomyoma. *Am J Physiol*. 1999;276(6, Pt 1):E1112–1118.
38. **Fuchs-Young R, Howe S, Hale L, Miles R, Walker C.** Inhibition of estrogen-stimulated growth of uterine leiomyomas by selective estrogen receptor modulators. *Mol Carcinog*. 1996;17(3):151–159.
39. **Gakhar G, Wight-Carter M, Andrews G, Olson S, Nguyen TA.** Hydronephrosis and urine retention in estrogen-implanted athymic nude mice. *Vet Pathol*. 2009;46(3):505–508.
40. **Leavitt WW, Takeda A.** Hormonal regulation of estrogen and progestin receptors in decidual cells. *Biol Reprod*. 1986;35(2):475–484.
41. **Taveira-DaSilva AM, Hathaway O, Stylianou M, Moss J.** Changes in lung function and chylous effusions in patients with lymphangioleiomyomatosis treated with sirolimus. *Ann Intern Med*. 2011;154(12):797–805, W-292–293.
42. **Malhowski AJ, Hira H, Bashiruddin S, et al.** Smooth muscle protein-22-mediated deletion of Tsc1 results in cardiac hypertrophy that is mTORC1-mediated and reversed by rapamycin. *Hum Mol Genet*. 2011;20(7):1290–1305.
43. **McCormack FX, Inoue Y, Moss J, et al.** Efficacy and safety of sirolimus in lymphangioleiomyomatosis. *N Engl J Med*. 2011;364(17):1595–1606.
44. **Bissler JJ, McCormack FX, Young LR, et al.** Sirolimus for angiomyolipoma in tuberous sclerosis complex or lymphangioleiomyomatosis. *New Engl J Med*. 2008;358(2):140–151.
45. **Taillé C, Borie R, Crestani B.** Current management of lymphangioleiomyomatosis. *Curr Opin Pulm Med*. 2011;17(5):374–378.
46. **Yu J, Parkhitko AA, Henske EP.** Mammalian target of rapamycin signaling and autophagy: roles in lymphangioleiomyomatosis therapy. *Proc Am Thorac Soc*. 2010;7(1):48–53.
47. **Kinder B, McCormack FX.** Clinical trials for rare lung diseases: lessons from lymphangioleiomyomatosis. *Lymphat Res Biol*. 2010;8(1):71–79.
48. **Walker CL.** Role of hormonal and reproductive factors in the etiology and treatment of uterine leiomyoma. *Recent Prog Horm Res*. 2002;57:277–294.
49. **Evans P, Brunsell S.** Uterine fibroid tumors: diagnosis and treatment. *Am Fam Physician*. 2007;75(10):1503–1508.
50. **Glasgow CG, Steagall WK, Taveira-Dasilva A, et al.** Lymphangioleiomyomatosis (LAM): molecular insights lead to targeted therapies. *Respir Med*. 2010;104(suppl 1):S45–S58.
51. **Matsui K, Takeda K, Yu ZX, et al.** Downregulation of estrogen and progesterone receptors in the abnormal smooth muscle cells in pulmonary lymphangioleiomyomatosis following therapy. An immunohistochemical study. *Am J Respir Crit Care Med*. 2000;161(3, Pt 1):1002–1009.
52. **Wahedna I, Cooper S, Williams J, Paterson IC, Britton JR, Tattersfield AE.** Relation of pulmonary lymphangio-leiomyomatosis to use of the oral contraceptive pill and fertility in the UK: a national case control study. *Thorax*. 1994;49(9):910–914.
53. **Brunelli A, Catalini G, Fianchini A.** Pregnancy exacerbating unsus-

pected mediastinal lymphangioleiomyomatosis and chylothorax. *Int J Gynaecol Obstet*. 1996;52(3):289–290.
54. **Cohen MM, Freyer AM, Johnson SR.** Pregnancy experiences among women with lymphangioleiomyomatosis. *Respir Med*. 2009;103(5):766–772.
55. **Yu J, Astrinidis A, Howard S, Henske EP.** Estradiol and tamoxifen stimulate LAM-associated angiomyolipoma cell growth and activate both genomic and nongenomic signaling pathways. *Am J Physiol Lung Cell Mol Physiol*. 2004;286(4):L694–L700.
56. **Marino M, Galluzzo P, Ascenzi P.** Estrogen signaling multiple pathways to impact gene transcription. *Curr Genomics*. 2006;7(8):497–508.
57. **Hoekstra AV, Sefton EC, Berry E, et al.** Progestins activate the AKT pathway in leiomyoma cells and promote survival. *J Clin Endocrinol Metab*. 2009;94(5):1768–1774.
58. **Ishikawa H, Ishi K, Serna VA, Kakazu R, Bulun SE, Kurita T.** Progesterone is essential for maintenance and growth of uterine leiomyoma. *Endocrinology*. 2010;151(6):2433–2442.
59. **Sawicka EH, Morris AJ.** A report of two long-surviving cases of pulmonary lymphangioleiomyomatosis and the response to progesterone therapy. *Br J Dis Chest*. 1985;79(4):400–406.
60. **Poh SC, Wang YT.** Lymphangioleiomyomatosis—treatment with progesterone. *Singapore Med J*. 1991;32(4):258–261.
61. **Bittmann I, Rolf B, Amann G, Löhrs U.** Recurrence of lymphangioleiomyomatosis after single lung transplantation: new insights into pathogenesis. *Hum Pathol*. 2003;34(1):95–98.
62. **Rege AS, Snyder JA, Scott WJ.** Benign metastasizing leiomyoma: a rare cause of multiple pulmonary nodules. *Ann Thorac Surg*. 2012;93(6):e149–e151.
63. **Goto T, Maeshima A, Akanabe K, et al.** Benign metastasizing leiomyoma of the lung. *Ann Thorac Cardiovasc Surg*. 2012;18(2):121–124.
64. **Walker CL, Hunter D, Everitt JI.** Uterine leiomyoma in the Eker rat: a unique model for important diseases of women. *Genes Chromosomes Cancer*. 2003;38(4):349–356.
65. **Kwiatkowski DJ.** Animal models of lymphangioleiomyomatosis (LAM) and tuberous sclerosis complex (TSC). *Lymphat Res Biol*. 2010;8(1):51–57.
66. **Kwiatkowski DJ, Zhang H, Bandura JL, et al.** A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells. *Hum Mol Genet*. 2002;11(5):525–534.
67. **Kobayashi T, Minowa O, Sugitani Y, et al.** A germ-line Tsc1 mutation causes tumor development and embryonic lethality that are similar, but not identical to, those caused by Tsc2 mutation in mice. *Proc Natl Acad Sci USA*. 2001;98(15):8762–8767.
68. **Onda H, Lueck A, Marks PW, Warren HB, Kwiatkowski DJ.** Tsc2(+/−) mice develop tumors in multiple sites that express gelsolin and are influenced by genetic background. *J Clin Invest*. 1999;104(6):687–695.
69. **Liu F, Lunsford EP, Tong J, et al.** Real-time monitoring of tumorigenesis, dissemination, drug response in a preclinical model of lymphangioleiomyomatosis/tuberous sclerosis complex. *PloS one*. 2012;7(6):e38589.
70. **Goncharova EA, Goncharov DA, Fehrenbach M, et al.** Prevention of alveolar destruction and airspace enlargement in a mouse model of pulmonary lymphangioleiomyomatosis (LAM). *Sci Transl Med*. 2012;4(154):154ra134.